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Human Serum Albumin And Hydroxy Ethyl Starch Are Protective Alone And In Combination For Human Hematopoietic Stem Cells Stored at -80ºC With 5% Dimethylsulphoxide

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Abstract

The preservation of hematopoietic stem cells (HSC), which allows postponing of their transplantation is by cryoprotection. Dimethyl sulfoxide (DMSO) is a widely used intracellular cryoprotectant which is toxic for cells and patients at temperature above 0 ºC. A possible approach to reduce these toxicities is with addition of extracellular cryoprotectors as hydroxyethyl starch (HES) and plasma proteins to allow the use of DMSO in lower final concentration. We tested the protective role of HES and the plasma protein human serum albumin (HSA), a for human hematopoietic stem cells, subjected either to osmotic stress (a

major factor in cellular injury during slow freezing) or to cryopreservation by unprogrammed freezing and storage at -80ºC. The viability was tested by trypan-blue exclusion assay.

HES has a protective effect on HSCs both against osmotic stress and cryopreservation and reduces cell aggregation. Addition of HSA to the cryoprotective solution improves viability and reduces cell clumping after thawing. Cryopreservation of HSCs with final 5% DMSO concentrations can be optimized by the addition of extracellular agents such as HES and HSA. This reduces DMSO toxicity to both HSCs and to patients during transplantation.

Keywords: DMSO concentration, extracellular cryoprotectors, -80 degrees, hydroxyethylstarch, HES, human albumin, HAS, hematopoietic stem cells, HSCs

Introduction

Hematopoietic stem cells (HSC) have intensive metabolism, therefore their long-term storage is possible only if they are frozen. Storage by freezing stem cells is an important part of most of the autologous and in some of the allogeneic hematopoietic stem cell transplantations. The major cryoprotectant, preferred for its quick universal trans-membrane penetration, which is usually used in 10% final concentration for cryopreservation, is dimethyl sulfoxide (DMSO) [1]. DMSO, though, is toxic both to cells [2] and to patients [3-8] and higher concentrations and longer exposition increase its toxicity. To reduce these undesired effects this cryoprotectant is used with lower (5%) final concentration [3, 9]. Lower DMSO concentration and slow rate of cell freezing (such as uncontrolled cryopreservation in mechanical freezer) expose cells to osmotic stress and dehydration. To reduce these effects extracellular cryoprotectors are used. Better viability results and cell survival is found after combined cryopreservation with intra- and extracellular cryoprotectors [10, 11]. The presence of extracellular cryoprotectants by suppressing cell dehydration allows the cells to be frozen with lower cooling rates thus maintaining the viability after cryopreservation [12]. These effects result from changes in the viscosity of extracellular solution and its vitrification temperature [1]. Sugars and their derivatives are known as natural cryoprotectants in hardy plants and animals able to tolerate low temperatures [13-16]. An additional possible mechanism of preserving cellular integrity may be a direct interaction of these agents with the cell membrane [9, 17, 18]. Infrared spectroscopy shows hydrogen bonds between trechalose and cell membrane dipalmitoil phosphatidil choline, which probably stabilizes the cell

membrane during freezing [15]. An alternative to these monosaccharides are the polysaccharides. They have the advantage to lower the osmotic injury, and by remaining in the extracellular solution to increase its viscosity and enhance vitrification during cooling [19, 20]. Such a high molecular saccharide is hydroxyethil starch (HES) which is a strongly branched uncharged water-soluble synthetic polymer, based on amylopectin modification [1, 21]. Combination of HES with DMSO as a cryoprotectant for stem cells freezing has been found to give better results than sole DMSO after thawing [22].

We decided to test the protective effect of hydroxyethyl starch on highly packed hematopoietic stem cells, subjected to osmotic stress as one of the major factors for cell injury.

Despite its cryoprotective role HES is not a physiological polymer for the human organism and has some toxicities. They are related with the changes in viscosity and oxygen carrying capacity of the patients' blood after infusion [16, 23]. HES also increases the hemorrhage risk probably due to dilution of blood clotting factors [24], and may lead to transient renal dysfunction, especially in septic patients [25]. There are studies showing that higher protein concentration in the frozen suspension improves the viability [26, 27]. This has led to the development of cryoprotective solutions, containing different concentrations of plasma proteins such as human serum albumin (HSA) [28]. HSA enhances the viscosity of the solution [29]. In an attempt to reduce the quantity of HES to be infused to the patients, we tested the effect human serum albumin in cryopreserving solution on cellular viability after freezing and thawing.

Materials and methods:

Apheresis-derived hematopoietic stem cell suspension was concentrated by platelet rich plasma removal after centrifugation at 1280G for 8 min. The samples of the packed cells were divided in two groups. In the first group one part of the cell suspension was mixed with four parts of saline solution of hydroxyethyl starch (HES) with relative molecular weight (450,000). The suspension was additionally diluted with 5% HES solution in distilled water thus achieving different concentrations of sodium chloride (NaCl) (0,9% , 0,77%, 0,45% and 0,28%) while preserving the same HES concentration.

In the second group one part of packed cells was mixed with four parts saline and diluted with distilled water achieving same sodium chloride concentrations (0,9% , 0,77%, 0,45% and 0,28%) like in the first group but reducing the HES concentration.

Both groups of samples were incubated at room temperature and viability was tested after 2, 24 and 48 hours.

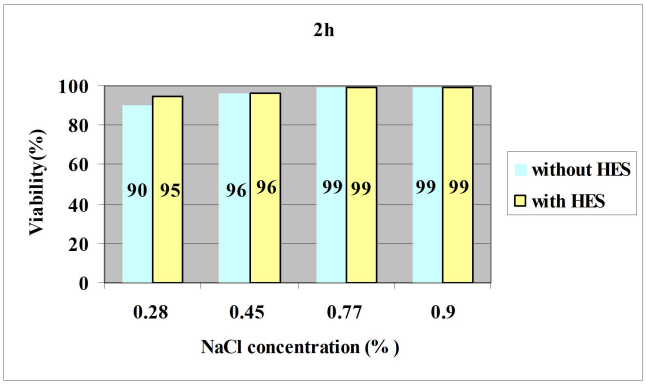
Other group of samples of apheresis-derived HSCs after packing were mixed with cryoprotective solution containing final concentration 5% of DMSO, 3% of HSA and 3.6 % of HES, subjected to uncontrolled freezing and storage at -80º C. After thawing the cell suspension was mixed with 5% DMSO saline solution with or without 2% human albumin, achieving serial dilution 1:2, 1:4 and 1:10, respectively.

The viability of the cells was tested by a trypan blue exclusion assay.

Results:

Exposure to osmotic stress reduces cell viability. Reduction is more pronounced at low osmolarity or longer incubation (Figure 1). In 0.77% NaCl the presence of 5% HES increases the viability with 6 % or 23 % after 24 h and 48 h incubation, respectively. The protective effect is most pronounced at the 0.28 % NaCl final concentration. In the presence of the polymer, the viability is higher with 24% and 36% after 24 and 48 hours incubation respectively.

Additionally, the presence of HES prevents cell aggregation – an effect, which is most visible in 0,28 % NaCl when the polymer is absent (figure 2).



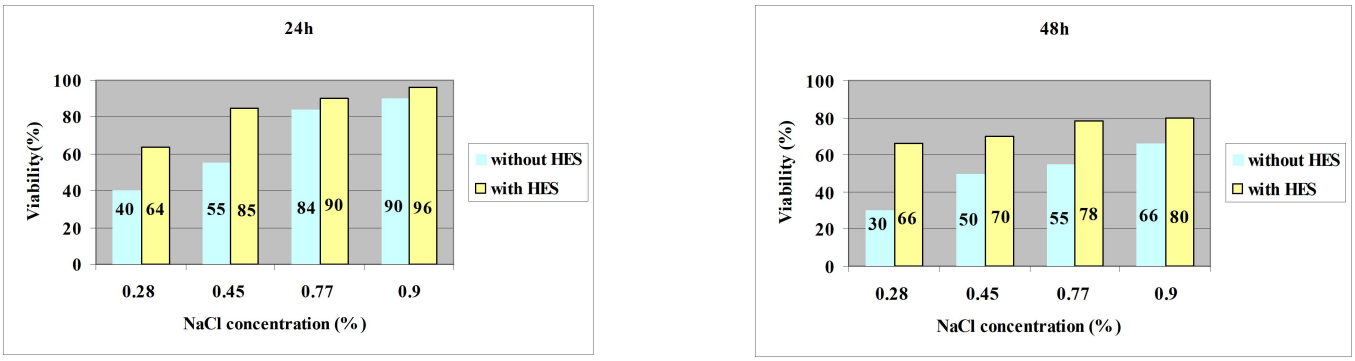
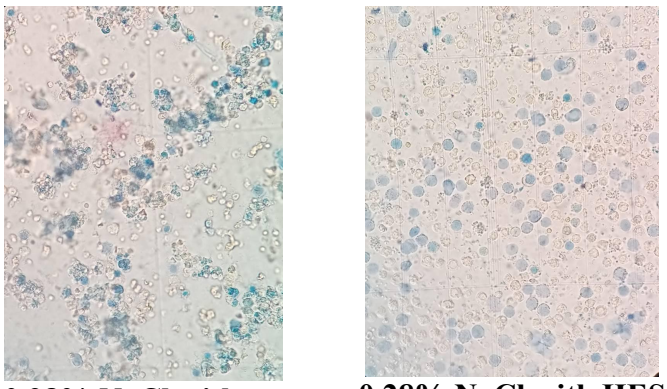


Figure 1 Cell viability after mixing with hypotonic solution in presence or absence of HES after 2, 24 and 48 hours



0,28% NaCl without HES **0,28% NaCl with HES**

Figure 2. Prevention of cell aggregation in hypotonic solution with HES after 24 hour incubation

The average viability of the cryopreserved cells after thawing and dilution with 5% DMSO saline solution decreases more prominently when the concentration of the extracellular cryoprotectors is lower (Table 1). The number of viable cells drops from 96.52% to 84.48% in the lowest HES concentration. The absence of human albumin in the diluting DMSO solution reduces the viable cells additionally with more than 11% to 72.77% (Table 1).

After dilution of the thawed cells, when the HES concentration is lower the presence of human serum albumin reduces the cellular death and aggregation.

Table 1 Viability of cryopreserved with 5% of DMSO, 3% of HSA and 3.6 % of HES hematopoietic stem cells at -80º after thawing and dilution with 5% saline solution of DMSO

Patient No	Standart viability	dilution 1:2 with albumin	dilution 1:4 with albumin	dilution 1:10 with albumin	dilution 1:2	dilution 1:4	dilution 1:10

1	99	99	98	90	92	91	70
2	99	90	87	93	85	60	75
3	98	91	87	70	75	73	53
4	99	99	80	70	70	45	40
5	99	99	96	90	94	80	70
6	97	80	85	97	69	72	69
7	93	94	97	100	na	na	na
8	99	98	97	74	97	83	73
9	98	99	99	86	98	67	71
10	98	98	98	70	98	98	58
11	97	88	65	83	70	57	80
12	91	90	80	80	83	72	78
13	98	90	84	89	86	77	88
14	93	68	66	86	71	60	74
15	95	95	87	87	81	83	90
16	96	96	75	78	91	78	79
17	97	89	89	96	83	85	88
18	94	99	77	75	97	70	69
19	97	99	86	89	97	75	84
20	99	92	89	88	83	87	83
21	94	87	87	82	81	87	79
Min	91	68	65	70	69	45	40
Max	99	99	99	100	98	98	90
Avg	96.52	91.61	85.78	84.48	84.91	74.68	72.77

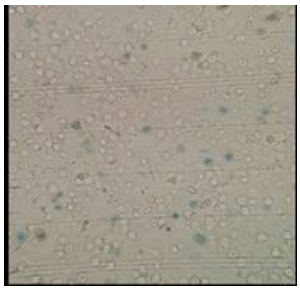
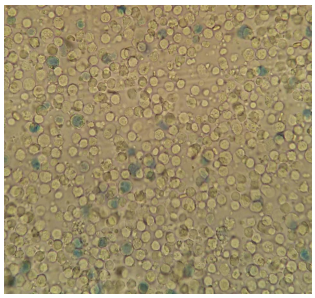
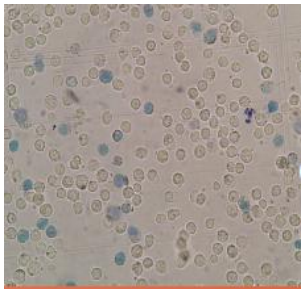
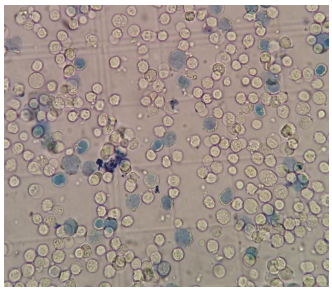
Patient I with albumin	Patient I no albumin	Patient II with albumin	Patient II no albumin
			

Figure 3. Prevention of cell aggregation by HSA after 24 hour incubation

Discussion:

Despite its cryoprotective role, DMSO is toxic for both the patients and the stem cells. Reduction of its toxicity mandates reducing the amount to be infused during transplantation. Lower concentration of DMSO in the cryoprotectant solution requires addition of extracellular cryoprotectants such as HES to maintain cellular viability. It has been demonstrated that HES slows dehydration of cells during freezing and stabilizes water molecules close to the cell membrane, thus reducing the osmotic stress [30]. Our results show reduction of HSC viability subjected to osmotic stress by exposure to hypotonic solutions. The addition of hydroxyethyl starch improves viability even after hypotonic exposure at 22ºC for 24 or 48 hours. Since HES is non-physiological substance for humans and may lead to undesired effects [16, 23-25], we reduce its final concentration in the cryoprotective solution by adding human serum albumin, which is another macromolecule known to have cryoprotective effect[29]. Addition of extracellular agents such as HES and human serum albumin to the cryoprotective solution offers additional advantages. DMSO, even at 5% final concentration, is toxic for HSCs for brief exposure at 22ºC or 37ºC after thawing [31]. Our results show that the presence of HES and HSA in the cell suspension after thawing reduces these toxic effects. Reduction of these agents leads to reduction of cell viability.

Our results show that with 5% DMSO higher concentrations of both extracellular cryoprotectants hydroxyethyl starch and human serum albumin maintain higher viability of hematopoietic stem cells and at the same time reduce the risk of aggregation and possible cell loss during infusion.

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